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Cell Donor Influences Success of Producing Cattle by Somatic Cell Nuclear Transfer

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ABSTRACT

To assess sources of variation in nuclear transfer efficiency, bovine fetal fibroblasts (BFF), harvested from six Jersey fetuses, were cultured under various conditions. After transfection, frozen-thawed lung or muscle BFF donor cells were initially cultured in DMEM in 5% CO_2 and air and some were transferred to MEM, with 5% or 20% O_2 or 0.5% or 10% serum and G418 for 2–3 wk. Selected clonal transfected fibroblasts were fused to enucleated oocytes. Fused couplets (n = 4007), activated with ionomycin and 6-dimethylaminopurine, yielded 927 blastocysts, and 650 were transferred to 330 recipients. Fusion rate was influenced by oxygen tension in a fetus-dependent manner ($P < 0.001$). Blastocyst development was influenced in a number of ways. Hip fibroblast generated more blastocysts when cultured in MEM ($P < 0.001$). The influence of serum concentration was fetus dependent ($P < 0.001$) and exposing fibroblast to low oxygen was detrimental to blastocyst development ($P < 0.001$). Cells from two of the six fetuses produced embryos that maintained pregnancies to term, resulting in eight viable calves. Pregnancy rates 56 days after transfer for the two productive donor fetuses, was at least double that of other recipients and may provide a fitness indicator of BFF cell sources for nuclear transfer. We conclude that a significant component in determining somatic cell nuclear transfer success is the source of the nuclear donor cells.

early development, embryo

INTRODUCTION

Since the first convincing demonstration of somatic cell nuclear transfer [1] and production of the first transgenic cattle [2], there has been a renewed interest in both understanding the concept of nuclear reprogramming and developing more efficient ways of producing animals by nuclear transfer. The potential benefit of improving genetic merit through the use of somatic cell nuclear transfer is being debated [3, 4]. However, recently published data leave little doubt that somatic cell nuclear transfer may be one of the most cost-effective approaches for introducing new, specific, genetic information into cattle [5].

Apparently, the key to successful somatic cell nuclear transfer is proper coordination of ooplasm and donor cell cycle [6]. Though there has been some dispute in the literature regarding which donor cell's cell cycle stages are optimum [2], there has been little disagreement that various combinations of donor cell stage and oocyte age result in live healthy offspring [5, 7, 8].

The vast majority of bovine somatic cell nuclear transfer studies have been initiated with oocytes derived from ovaries collected at slaughter, though in vivo-derived oocytes have also served as starting material [9]. In vitro maturation of oocytes, well established before the advent of somatic cell nuclear transfer, has been adopted with little change [10–13]. Most attempts to improve the cloning process in cattle have focused on methods of reconstitution [10], donor cell types [10, 14–18], activation of couplets [19, 20], and, to a lesser extent, embryo culture [21–23].

Recently, several strategies to increase speed of embryo manipulation and to decrease required skill level have been reported [24–26]. All three of these approaches reduce specialized equipment needs, in one case, dramatically [26].

The potential of various cell types to serve as nuclear donors, such as cumulus [27], embryonic stem (ES) cells [28], fetal and adult fibroblasts [14], granulosa [29], myoblast [30], neurons [31], and Sertoli cells [32] has been evaluated. To date, no particular cell type has an overwhelming advantage over another. When fetal and adult fibroblasts have been compared, fetal fibroblasts make superior donor cells. Lower passage cells have an advantage over higher passages cells [15, 33–35]. Thus, the number of divisions after the initial mitosis appears to be an important factor in determining a cell's usefulness as a nuclear donor [36].

Beyond evaluating cell types and manipulating serum concentrations in an effort to control the stage of the cell cycle, little attention has been focused on optimizing culture conditions of donor cells. In the study reported here, fibroblasts from six fetuses served as nuclear donor cells. Their clonal growth under G418 selection and ability to generate blastocysts and produce calves was assessed. Fetal fibroblasts from lung and muscle tissue were compared. In addition, during clonal growth, the influences of culture media and serum and oxygen concentrations were evaluated. Of the parameters studied, the fetus from which the fibroblasts were harvested had the greatest influence on efficiency of producing calves.

MATERIALS AND METHODS

Bovine Fetal Fibroblast Harvest

Jersey females were inseminated with semen from one of three bulls to produce six fetuses (fetuses 7, 8, and 9 from bull A, fetus 10 from bull B, and fetuses 11 and 12 from bull C semen). Fetuses were aseptically collected at slaughter and ranged in gestational age from 62 to 107 days (fetus 7, 101 days; fetus 8, 62 days; fetus 9, 68 days; fetus 10, 95 days; fetus 11, 107 days; and fetus 12, 99 days). After removal of the skin, approximately 1 g of muscle from the biceps femoris was minced in 5 ml of 0.05% trypsin with 0.53 mM EDTA and incubated for 1 h in 5% CO_2 in air at 38.5°C. An equal volume of high glucose Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum (FCS), 4 mM gluta-

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mine, 50 U/ml of penicillin and 50 μg/ml streptomycin was added and the cells were centrifuged at 800 × *g* for 10 min. Pellets were resuspended in fresh DMEM and centrifuged again, after which the cells were transferred into three T75 flasks and cultured at 5% CO_2 in air at 38.5°C. Primary muscle fibroblast cultures were passaged either once or twice before being frozen in 92% FCS and 8% dimethyl sulfoxide (DMSO). Cells were passaged when the cells reached 80% to 90% confluence, usually taking 3–4 days. There were minor growth rate differences between fetuses. Bovine fetal fibroblasts from fetuses 7 and 10 were frozen at passage 1 and BFF from the remaining fetuses were frozen after two passages. Lung fibroblasts were also harvested from fetuses 7, 8, and 10. Lung BFF from fetus 7 was frozen at passage 1 and those from fetuses 8 and 10 were frozen at passage 2.

Bovine Fibroblast Transfection and Selection

Prior to use as nuclear donor cells, BFF were thawed and cultured for 2–5 days in DMEM with 10% FCS. An aliquot (400 μl) of single-cell suspension containing approximately 1×10^7 cells/ml (4×10^6 cells) was transfected with 10 μg of a transgene construct containing genes for neomycin resistance (neo), green fluorescent protein (GFP), and a peptidoglycan hydrolase (lysostaphin) with expression directed to mammary gland secretory epithelium [37]. The neo coding sequence, driven by the SV40 large T antigen regulatory element, was derived from pEGFP-N1 (Clontech, Palo Alto, CA) for expression in BFF during cell culture. The human elongation factor 1a promoter was used to express nuclear localized, enhanced GFP gene during the blastocyst stage of development [14, 38]. Transfection was achieved in an electroporation cuvette with 4-mm gap width (400 V, 500 μF; Bio-Rad Pulsar II, Hercules, CA). After transfection cells, were cultured for 48 h in DMEM in 100-mm tissue culture dishes. Selection for stable integrants was initiated by addition of G418 (400 μg/ml). Cells remained under selection and were refed every 7 days with G418-containing medium. Colonies in four or five replicate plates were counted and examined for GFP fluorescence on the 19th day of culture, following initiation of G418 selection (21 days after transfection). Selected GFP-expressing colonies were isolated with an 8-mm cloning cylinder. The cells intended as nuclear donors were harvested by washing twice with Ca^{2+}- and Mg^{2+}-free PBS (plus 0.05% trypsin). Isolated cells were diluted in TL-HEPES with 10% FCS, removed from cloning cylinders, and centrifuged at 800 × *g* for 10 min. Pellets were resuspended in TL-HEPES and transferred to manipulation drops for nuclear transfer.

Three experiments were designed to compare the effectiveness of BFF isolated from muscle and lung in somatic cell nuclear transfer and to assess the influence of culture media, serum concentration, and oxygen concentration.

Experiment 1. Bovine fetal fibroblasts were isolated from hip or lung tissues and cultured in either minimum essential medium (MEM) or DMEM in a 2 × 2 factorial design. Transfected hip and lung BFF were selected with G418 (400 μg/ml) in either DMEM with 10% FCS in 5% CO_2 in air or MEM with 10% FCS in 5% CO_2 in air for 18–20 days until they were used as nuclear donors. The usefulness of these BFF as nuclear donors in the production of blastocysts was evaluated. Grade 1 and 2 blastocysts were transferred to available recipients.

Experiment 2. The influence of serum starvation during G418 selection was tested. Transfected hip BFF were cultured in MEM or DMEM with 10% FCS and 5% CO_2 in air during the first 14 days of selection. On Day 14, one half of the dishes were fed as usual with 10% FCS in appropriate media and the other half were fed with 0.5% FCS. G418 selection was continued in 5% CO_2 in air for an additional 4–6 days, until the cells were used for nuclear transfer. Only BFF grown in MEM were used for nuclear transfer (NT). Blastocyst development was assessed, and grade 1 and 2 blastocysts were transferred to available recipients.

Experiment 3. Transfected BFF were subjected to oxygen concentrations of 5% or 20% (air) during G418 selection in a modified crossover experimental design. Hip BFF were grown in MEM with 10% FCS in either 5% CO_2 in air (high O_2) or 5% CO_2 + 5% O_2 + 90% N_2 (low O_2) before and after transfection (high O_2 before and after transfection; low O_2, before and after transfection). Hip BFF in dishes were also cultured in high or low O_2 concentrations and then switched to the other concentration after transfection (high O_2 before, low O_2 after transfection; low O_2 before, high O_2 after transfection). During the last 5 days of selection, serum concentration was reduced to 0.5% for all groups.

Clonal growth was assessed in all three experiments. Dishes were stained with R-250 Coomassie blue stain (1.25 mg/ml) + 10% glacial acetic acid + 50% methanol for 10 min on Day 28 posttransfection. After two washes, dishes were air dried and colonies were counted.

Approximately half of the blastocysts generated in these experiments, along with 120 additional blastocysts were transferred to recipients in an attempt to produce calves. The additional embryos were generated as described in these experiments but were not included in the analyses because they did not have a comparison group for a variety of technical reasons (not enough oocytes on a given day, contamination of a treatment group, not enough recipients, etc.).

Oocyte Harvest, Enucleation, and Nuclear Transfer

Oocytes were purchased from a commercial supplier (BoMed; Madison, WI) and matured in transit in M199 + LH and FSH. Additionally, ovaries were purchased from a local slaughterhouse (MoPAC, Allentown, PA) and matured in the laboratory using a protocol similar to that previously described [39]. Oocytes from BoMed were only used for in vitro studies, while oocytes from MoPAC were used for both in vitro and in vivo studies. Briefly, ovaries were washed in 1% Nolvasan in 0.9% saline and transported to the laboratory at room temperature within 3–5 h after slaughter. Four- to 8-mm follicles were sliced [40] with scalpel blades and the ovaries were shaken in beakers containing 150 ml of HEPES-buffered Tyrodes lactate solution (TL-HEPES, BioWhittaker, Inc., Walkerville, MD). The oocytes were washed in Em-Con filters (model #04135; ImmunoSystems, Inc., Scarborough, ME). Cumulus-oocyte complexes were placed in culture wells, pipetted to partially remove cumulus, which was left in the well, then matured in Ham F-10 with 10% FCS, penicillin, streptomycin, and LH [11]. Seventeen hours after the initiation of maturation, oocytes were vortexed in 1 ml of 0.9% saline containing 1% hyaluronidase and 10% PVP for 4 min, washed in Dulbecco PBS (D4031; Sigma-Aldrich, St. Louis, MO) with 1% BSA (A3311; Sigma) and evaluated for polar bodies. Oocytes were cultured further in Ham F-10 with 10% FCS until enucleated 18–20 h after initiation of maturation.

Just before enucleation, oocytes were exposed to cytochalasin B and Hoechst 33342 DNA stain in TL-HEPES for 20 min and then transferred to TL-HEPES with 1% BSA. Oocytes were manipulated at room temperature and removal of the metaphase plate was confirmed by briefly exposing the enucleation needle to fluorescent light (350-nm excitation, 450-nm bandpass filters). Enucleated oocytes (cytoplasts) were returned to maturation medium. Within several hours after enucleation, fibroblasts were inserted into the perivitelline space of cytoplasts to form couplets. Once 10–15 couplets were prepared, they were immediately transferred to electrofusion solution (270 mM mannitol, 50 μM $MgCl_2$). After a short equilibration (less than 10 min), the couplets were transferred to a fusion chamber with a 1.0-mm gap between electrodes, mechanically aligned, and fused with a single DC pulse of 115-V magnitude and 42-μsec duration (BTX 200, Inc., Hawthorne, NY).

Embryo Culture and Transfer

Couplets were then transferred to CR1aa media [41]. One to 4 h after fusion, couplets were activated in 5 nM Ionomycin (Sigma-Aldrich) in CR1aa for 4 min followed by a 4-h exposure to 1.9 mM 4-dimethylaminopyridine (Sigma-Aldrich) in CR1aa. After activation, the fused couplets were washed in CR1aa and cultured for approximately 16 h before being transferred to BARC-1 [38] in 5% CO_2 + 5% O_2 + 90% N at 38.5°C for 7 days. Blastocysts from each treatment were counted and GFP expression evaluated before embryo transfer. Estrus-synchronized 14- to 17-mo-old Holstein heifers received two grade 1 or 2 blastocysts on Day 7 or 8 of their estrous cycle. Parthenogenotes served as activation controls for each day and in vitro-fertilized embryos served as media controls. Return to estrus was monitored with the aid of the Heat Watch system (DDX, Inc., Denver, CO). Ongoing pregnancies were observed by ultrasound on Days 39, 56, and 69 posttransfer. Fluid detected in the uterus was taken as an initial indicator of pregnancy. Heartbeats were observed by Day 56.

All procedures involving live animals were conducted in accordance with the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* and approved in advance by the Beltsville Agricultural Research Center's Animal Care and Use Committee.

Data Analysis

All statistical analyses were performed with SPSS version 10 software (SPSS, Inc., Chicago, IL). Least-square means and their standard errors are reported. Fused couplets served as the denominator in calculating blastocyst development rates and as a weighting factor in some analyses. Blastocyst percentages were first arcsine transformed before being subjected to analysis of variance. Fetuses from which fibroblasts were derived were always a factor in the statistical model. Other factors such as source tissue,

TABLE 1. Blastocyst development rate of nuclear transfer embryos produced with donor fibroblasts cultured in DMEM or MEM from three fetuses.

	Fetus 7		Fetus 8		Fetus 10	
Media	Fused*	Blastocysts†	Fused	Blastocysts	Fused	Blastocysts
DMEM	372	23.6 ± 0.9^a	292	14.3 ± 1.0^a	311	23.9 ± 0.9^a
MEM	355	24.8 ± 0.9^a	281	19.7 ± 0.9^b	319	23.0 ± 0.9^a

* Number of successfully fused couplets.
† Least-square mean blastocyst development rate ([no. blastocysts/no. fused couplets] × 100) ± SEM.
[a,b] Column means with different superscripts differ significantly ($P = 0.004$).

fibroblast culture media, oxygen, and serum concentration were included when appropriate. Pregnancy rates were compared by chi-square analysis.

RESULTS

Experiment 1. Influence of Culture Media and Fibroblast Tissue Source

Blastocyst development was evaluated in a 2 × 3 × 2 factorial experimental design. The ability of fibroblasts isolated from hip muscle or lung tissue of four fetal fibroblast sources cultured in MEM or DMEM were compared for their ability to form colonies, express GFP, and produce blastocysts when used as nuclear donor cells.

Lung tissue from fetuses 7, 8, and 10 produced more colonies than hip-derived fibroblasts when enumerated on Day 28 of culture (8.3 ± 0.3 vs. 3.1 ± 0.2 colonies/plate), but hip fibroblasts from fetus 9 were more proliferative than lung fibroblasts (1.2 ± 0.07 vs. 5.3 ± 0.7 colonies, fetus × tissue interaction, $P < 0.001$). Fetuses 7, 8, and 9 generated a higher proportion of GFP-expressing colonies from hip fibroblasts than from lung fibroblasts (59% ± 2% vs. 29% ± 3% green colonies/plate), while lung tissue from fetus 10 generated a higher percentage of green colonies than did hip fibroblasts (49 ± 3% vs. 67 ± 5%, fetus × tissue interaction, $P < 0.001$). An interaction between source tissue type and culture media on proportion of GFP-expressing colonies was also observed; lung fibroblasts expressed GFP more frequently when cultured in DMEM while hip-derived fibroblasts produced a higher proportion of GFP colonies when cultured in MEM.

Couplet fusion rate was only moderately influenced by the factors in this study. Fibroblasts derived from the hip tissue of fetus 10 tended to fuse more effectively than fibroblasts from lung (84% vs. 73% fusion, $P = 0.048$). No other influence on fusion rate by fetus, tissue type, or media was observed (data not shown).

When development to blastocysts was considered, fibroblasts derived from hip muscle had a clear advantage. On average, hip muscle fibroblasts resulted in 50% more blastocysts than did fibroblasts derived from lung tissue (26.1% ± 0.5%, n = 978 vs. 17.0% ± 0.6%, n = 901, $P < 0.001$). The ability of hip and lung fibroblasts to produce blastocysts were not influenced by the fetus from which they were derived ($P = 0.500$).

Fibroblasts from fetus 8 produced a higher proportion of blastocysts when cultured in MEM than when cultured in DMEM, whereas culture media did not influence blastocyst development from fetus 7 or 10 fibroblasts (Table 1).

An interaction was also observed between culture media and tissue source. Fibroblasts derived from hip muscle produced blastocysts at a higher rate if the fibroblasts were cultured in MEM. Alternatively, lung-derived fibroblasts produced over 30% more blastocysts if they were cultured in DMEM (Table 2).

TABLE 2. Blastocyst development rate of nuclear transfer embryos produced with fibroblasts derived from fetal hip or lung tissue of fetuses 7, 8, and 10 cultured in DMEM or MEM.

	Hip fibroblasts		Lung fibroblasts	
Media	Fused*	Blastocysts†	Fused	Blastocysts
DMEM	481	21.3 ± 0.8^a	494	19.8 ± 0.8^a
MEM	497	30.9 ± 0.7^b	458	14.1 ± 0.8^b

* Number of fused couplets.
† Least-square mean blastocyst development rate ([no. blastocysts/no. fused couplets] × 100) ± SEM.
[a,b] Column means with different superscripts differ ($P < 0.001$).

Experiment 2. Influence of Serum Concentration, Culture Media, and Fetal Fibroblast Source

The debate in the scientific literature as to whether donor cell serum starvation is advisable, coupled with our observation that serum starvation appears to compromise the health of fibroblasts, led us to compare colonial growth and transgene expression at two serum concentrations in two fibroblast culture media. Blastocyst development from hip fibroblasts from three fetuses served as nuclear donor cells and MEM was used as the fibroblast culture medium.

In this second experiment, fibroblasts from fetus 11 produced the most colonies/plate (7.9 ± 0.5) and fibroblasts from fetus 7 the fewest (3.2 ± 0.5, $P < 0.001$). A statistically significant interaction was observed on colonial growth between serum concentration and medium. Cells grown in DMEM with low serum proliferated poorly and somewhat unpredictably (1.0 ± 1.0 colonies/plate), whereas fibroblasts grown in MEM with 10% serum generated the most colonies per plate (4.0 ± 0.4, serum × media interaction, $P = 0.017$). None of the treatment conditions influenced the proportion of fibroblast colonies that expressed GFP.

As in the first experiment, fusion rate was not influenced by treatment parameters studied. Fusion rates of couplets for fibroblasts incubated in 0.5% or 10% serum were identical at 74%.

Fetal fibroblast cell source affected the influence of serum (Table 3). Serum concentration for fibroblasts from fetus 7 had no effect on blastocyst development. In contrast, blastocyst rate was highest when fibroblasts were incubated in 10% serum for fetus 10 and in 0.5% serum for fetus 11 during the final week of culture.

Experiment 3. Influence of Oxygen Tension

In the final series of experiments, the influence of oxygen tension on colony formation and transgene expression of fibroblasts was tested in a crossover experimental design. Subsequently, blastocyst development was evaluated in a factorial design with fetus and O_2 tension (atmospheric and low) as main effects.

Colony propagation was greatest when fibroblasts were

TABLE 3. Blastocyst development rate of embryos produced with fibroblasts cultured in MEM with either 0.5% or 10% fetal calf serum for 6 days prior to nuclear transfer from three fetuses.

Serum concentration (%)	BFF 7		BFF 10		BFF 11	
	Fused*	Blastocysts†	Fused	Blastocysts	Fused	Blastocysts
0.5	130	22.3 ± 0.9^a	190	12.6 ± 1^a	182	37.9 ± 0.9^a
10	190	18.9 ± 0.9^a	192	19.3 ± 1^b	184	19.6 ± 0.9^b

* Number of fused couplets.
† Least-square mean blastocyst development rate ([no. blastocysts/no. fused couplets] × 100) ± SEM.
[a,b] Column means with different superscripts differ ($P < 0.001$).

cultured in 20% O_2 and then switched to 5% O_2 following transfection (5.8 ± 0.4 colonies/plate, $P < 0.001$). The other three treatments produced fewer colonies and did not differ from one another.

Fibroblasts from fetus 10 produced almost twice as many colonies (5.6 ± 0.4 colonies/plate) as did fibroblasts from fetuses 7 (3.3 ± 0.4 colonies/plate) and 12 (3.2 ± 0.4 colonies/plate, $P < 0.001$). The highest proportion of GFP-expressing colonies per plate resulted from fetus 7 cells (75% ± 6%; fetus 10, 57% ± 8%; fetus 12, 41% ± 5%, $P < 0.001$). The four oxygen tension treatments did not influence the proportion of GFP-expressing colonies.

In an initial pilot study (n = 152 fused couplets), embryos produced from fibroblasts cultured in low or normal (air) oxygen tension were either cultured in BARC-1 or G1/G2 (Vitrolife, Denver, CO) media for 7 days. No differences in blastocyst development were attributable to embryo culture media ($P = 0.620$). Therefore, in all subsequent trials, the embryo culture media comparison was dropped and embryos were only cultured in the G1/G2 system.

Oxygen tension during fibroblast culture influenced both rates of couplet fusion and blastocyst development in a fetal fibroblast cell source-dependent manner. Fibroblasts from fetus 7 exhibited a higher fusion rate when grown in low oxygen tension (Table 4). However, the subsequent development of those embryos was extremely poor. While the oxygen tension in culture of donor fibroblasts had no influence on blastocyst development for fetus 10 fibroblasts, higher oxygen concentration was beneficial for blastocyst development for embryos derived from fetus 12 fibroblasts.

Embryo Transfer

From 4007 couplets, 920 good-quality blastocysts were generated, including 120 not produced in the experiments described above. Overall blastocyst production rate was lowest for embryos generated from fetus 8 fibroblasts and highest from fetus 11 fibroblasts (Table 5, $P < 0.001$). The eight live calves produced arose from BFF7 and BFF10 fibroblasts.

Eighty-two percent of potential pregnancies were lost within 56 days after transfer (Fig. 1). At that time, the pregnancy rate of recipients receiving embryos generated from BFF7 and BFF10 cells could be distinguished from pregnancy rates of the other recipients. Average pregnancy rate, at 8 wk, for embryos derived from BFF7 and BFF10 cells was higher (24%) than the mean pregnancy rate of recipients receiving embryos produced with cells from the other four fetuses (7%, $P < 0.001$).

The overall efficiency of producing calves, 1% of embryos transferred, was too low to provide adequate statistical power to assess the influence of the various fibroblast and embryo culture conditions tested in the three experiments. However, enough embryos were transferred to demonstrate that fibroblasts from different fetuses affect the calving rate (Table 5). The success rate of blastocyst production seemed to be unrelated to the ability of those embryos to survive to term in utero.

The superior in vitro performance of fibroblasts from fetuses sired by bull C (BFF 11 and 12) was not reflected in in vivo development rates. On the contrary, embryos derived from fetuses sired by bull A (BFF 7, 8, and 9) had substantially higher pregnancy rates than did embryos derived from bull C fetuses. All of the calves born alive were derived from fetuses sired by bulls A and B. As a result, there was a tendency for mean gestation length of recipients receiving embryos from fetal fibroblasts sired by bulls A and B to be longer than for those sired by bull C (sire A, 49.801 ± 4.256; sire B, 58.979 ± 5.814; sire C, 35.058 ± 7.941; $P = 0.053$). At 8 wk postestrus, the proportion of bull A recipients (recipients receiving embryos derived from fetuses sired by bull A) was 20% whereas bull C recipients was 2%. Bull B (fetus 10) recipient pregnancy rate was similar to bull A at 22%.

DISCUSSION

More than 900 good-quality blastocysts were generated in the above three experiments. About two thirds were transferred to embryo recipients. Because calving rates were low, it was not possible to make a rigorous assessment of the impact of in vitro treatments on overall somatic cell

TABLE 4. Couplet fusion and blastocyst development rates of embryos produced with fibroblasts cultured in either 20% (atmosphere) or 5% oxygen tension before use as nuclear donor cells from three fetuses.*

Fibroblast treatment	BFF 7		BFF 10		BFF 12	
	Fusion (%)†	Blastocyst‡	Fusion (%)	Blastocyst	Fusion (%)	Blastocyst
Atmosphere	112 $(68.8 \pm 0.9)^a$	26.0 ± 1.6^a	140 $(80.4 \pm 0.7)^a$	27.7 ± 12.3^a	174 $(83.6 \pm 0.7)^a$	38.1 ± 1.2^a
Low oxygen	111 $(77.3 \pm 0.9)^b$	3.5 ± 1.5^b	141 $(74.3 \pm 0.8)^b$	24.3 ± 1.3^a	171 $(74.9 \pm 0.8)^b$	16.0 ± 1.3^b

* Fibroblasts were cultured in either high O_2 (atmosphere) or low O_2 for 28 days, including 19 days after transfection.
† Number of fused couplets (least square mean fusion rate [no. fused couplets/no. couplets] × 100 ± SEM).
‡ Least-square mean blastocyst development rate ([no. blastocysts/no. fused couplets] × 100) ± SEM.
[a,b] Column means with different superscripts differ ($P < 0.001$).

TABLE 5. Summary of embryo transfer results.*

Fibroblast source			Fused couplets	Blastocysts†	Embryo Transfer Recipients	Pregnant at 56 days‡	Live calves	Postnatal survival§
Sire	Fetus	Sex						
A	7	Male	1257	23.1[cd]	109	25	4	2
A	8	Male	637	17.1[a]	40	13	0	0
A	9	Male	243	19.8[ab]	32	13	0	0
B	10	Female	1228	22.1[bc]	97	22	4	3
C	11	Female	366	29.7[e]	38	3	0	0
C	12	Female	276	25.3[de]	14	0	0	0
		Overall	4007	21.8	330	18	8	5

* Includes 120 nuclear transfer embryos produced in the same manner as those described in the three experiments but not included in other analyses.
† Mean blastocyst development rate ([no. blastocysts/no. fused couplets] × 100).
‡ Percentage of recipients still pregnant at 56 days posttransfer assessed by ultrasound.
§ Survival beyond 6 months.
a–e Values within a column with different superscripts are statistically different ($P < 0.001$).

nuclear transfer success. However, some general relationships have emerged that provide an opportunity to draw a few tentative conclusions.

In the first experiment, the influence of fibroblast culture media and fibroblast tissue source on in vitro development was assessed. Many somatic cell types such as cumulus or granulosa, oviductal, uterine, skin fibroblasts, liver cells, thymocytes, spleen cells, and macrophages have been used as nuclear donor sources in bovine nuclear transfer with no clear advantage except that cells from adults do not support in vitro or in vivo development as well as cells from neonates or fetuses [10, 27, 42]. For purposes of producing genetically engineered cattle, fetal fibroblasts are an appealing nuclear donor source because they readily survive in vitro culture. Fibroblasts are most commonly derived from lungs, muscle, and skin. Of the various sources of fibroblasts, lung fibroblasts are among the most prolific [43]. The use of lung fibroblasts as nuclear donors has been previously reported [44] but has not been directly compared with other fibroblast sources. In our hands, lung fibroblasts were demonstrably less efficient as nuclear donor sources for blastocyst production than muscle fibroblasts and were more difficult to manipulate than those derived from fetal muscle.

DMEM appears to be the most commonly used culture medium for somatic donor cells [14, 21, 45], though MEM is also a widely used synthetic cell culture medium [33, 46]

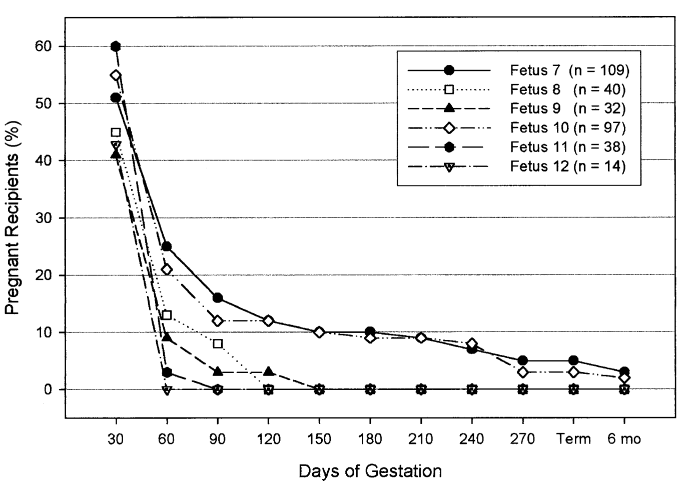

FIG. 1. Pregnancy profile of Holstein recipients into which confirmed transgenic somatic cell nuclear transfer blastocysts were transferred. Only recipients receiving embryos derived from fetuses 7 and 10 maintained gestation to term (n = number of recipients).

and was used in a modified form, GMEM, in the first reported somatic cell nuclear transfer study [1, 33, 46]. Tissue culture medium 199 has also been used to a lesser extent [33, 46–49]. In our direct comparison of MEM and DMEM, both media performed similarly across fetal fibroblast sources, but MEM was superior for culturing hip muscle fibroblast donor cells for blastocyst production (Tables 1 and 2). Therefore, MEM is now used as our standard media for fibroblast culture.

Manipulating serum concentration of fibroblast cultures before nuclear transfer was adopted as a means of synchronizing the cell cycle of the donor cell and cytoplast [50]. Blastocyst production rate was enhanced when fetal bovine cells were serum starved, but the advantage of serum starvation was not apparent for fibroblasts harvested from adults [16]. Others have found no benefit from serum starvation for enhancing cleavage or blastocyst production in the bovine [29] or porcine NT embryo development [51]. In the studies reported here, culturing fibroblasts in either 0.5% serum or 10% serum for the final week before use as nuclear donors had differential effects on blastocyst development depending on the fetal fibroblast source (Table 3). Fibroblasts from the three fetuses tested all responded differently to serum concentration. Performance of fibroblasts from the two fetuses that produced live young either exhibited no preference or produced more blastocysts when cultured in 10% serum. Because the three fetuses tested were sired by three different bulls, it is not possible to distinguish between a potential genetic response to serum concentration and differences between fetuses.

Though the literature and our study is equivocal regarding the benefits of serum concentration in fibroblast cultures before NT, the advantage of 10% serum has been clearly demonstrated in the rate of calf production from transfected fibroblasts [5]. In that study, transfected fibroblasts cultured in 10% serum and transferred to recipients resulted in a calving rate of 29%, as compared with 4% if the transfected fibroblasts were serum starved (0.5% serum) before use as nuclear donor cells [5]. It was also shown that, whereas 10% serum was beneficial to transfected fibroblasts, the opposite was true for nontransfected fibroblasts. The calving rate of nontransgenic clones was twice the rate in the serum-starved group compared with the 10% serum group. We have confirmed that finding with nontransfected BFF10 fibroblasts (data not shown).

Growth of mammalian cells in culture was enhanced and population doublings extended by approximately 25% when oxygen tension was reduced from 20% to 10% [52]. The length of telomere shortening, as a result of cell division, is influenced by oxidative stress. Atmospheric (20%) oxygen tension in fibroblast cultures can cause telomere shortening of more than four times that observed in low O_2 cultures [53]. Atmospheric oxygen concentration (in which most tissue cultures are conducted) is considerably higher than the oxygen tension found in most tissues, which is closer to 5% [43]. Cultured cells from monkey, mouse, and rat exhibited equal or improved colony formation under low (1–3%) oxygen tension [52, 54, 55]. However, the beneficial effects of low oxygen tension for cultured cells has not always been demonstrated [43, 56]. In a parallel study, plating efficiency (i.e., colony formation from single cells) of hip muscle fibroblasts from the same fetuses described in this report confirmed that low oxygen tension did not enhance colony formation [57]. However, as with the serum concentrations, the influence of oxygen tension during fibroblast culture on somatic cell nuclear transfer efficiency was fetus dependent. The three fetuses tested were sired by three different bulls. Therefore, it is not possible to distinguish between a fetus or genetic affect.

Over the three experiments, blastocyst development rate ranged from 17% to 30% (mean ± SEM, 22% ± 3%) for the six fetuses tested. Contemporaneous in vitro-produced and parthenogenote blastocyst development rates ranged from 2% to 63% (33% ± 2%) and 13% to 59% (36% ± 9%), respectively. There was a clear clustering in blastocyst production based on sire. Fetuses of bull C (BFF 11 and 12) produced nuclear transfer blastocysts most efficiently and those of bull A (BFF 7, 8 and 9) least efficiently. However, favorable in vitro development did not predict in vivo success. Fetuses of bull C produced no live offspring. It should be noted that only 104 embryos representing bull C were transferred (about half as many as for the other two bulls). Even so, based on the data presented here, we would have expected at least one calf from bull C. These observations suggest there may be a genetic component to the success of fetal cells as nuclear donors, but genetics that favor in vitro development may not be the most desirable.

These experiments were conducted over a 3-yr period. The overall strategy was to superimpose a new treatment on the best conditions defined in the previous experiment (Expt. 1, Hip, MEM; Expt. 2, 0.5% serum; Expt. 3, 20% O_2). So it may not be too surprising that our calving rate improved over time (Expt. 1, 1% of embryo transfers [ETs]); Expt. 2, 3% of ETs; Expt. 3, 10% of ETs, P = 0.015). However, the improvement with time may simply be attributable to our increasing competence with the process or random chance.

The 8-wk pregnancy rate may serve as a predictor of the success of bovine nuclear transfer experiments. The average pregnancy rate for recipients receiving embryos generated from BFF7 and BFF10, the two fetal fibroblast sources that produced live calves, was 23%. The aggregate pregnancy rate for the other four fetuses was 7%. From the work reported here, one would expect three or four pregnant recipients at 56 days from 15 to 20 embryo transfers from good fibroblast sources, and no pregnancies or possibly one pregnancy for embryos derived from fibroblast sources such as fetuses 8, 9, 11, and 12. Interestingly, the 8-wk pregnancy rate may not be universally predictive. In a similarly sized study, differences did not appear apparent until approximately 160 days of gestation [5].

There are few published studies that provide enough detail to determine if somatic cell nuclear transfer calving efficiencies observed are attributable to fibroblast cell lines or to a fetal effect or possibly a genetic effect. To try to ensure that we were comparing the influence of fetuses as the source of variation, we pooled all the cells harvested from a given fetus before aliquoting and freezing. The differences observed were therefore due to experimental variation, fibroblast treatments, or fetal fibroblast source. In our hands, the most profound differences in nuclear transfer efficiency were attributable to fetuses from which fibroblast were harvested. Though the data set is too small to be compelling, there is a suggestion that the genetic background of the fetus may contribute to the overall efficiency of producing somatic cell clones. The use of cells from fetuses produced by sires A and B resulted in pregnancy rates of 21% and 22%, respectively, at 56 days postestrus, but the pregnancy rate was only 2% for recipients receiving embryos generated from sire C fetuses.

These results support the notion that the source of the donor cells may be one of the most important factors in determining the success of a somatic cell nuclear transfer project [8] and suggest that it may be possible to make a reasonable assessment of the clonability of bovine nuclear transfer donor cells by 60 days posttransfer.

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REFERENCES

1. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature 1997; 385:810–813.
2. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell Scientific Publications C, Ponce dLF, Robl JM. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 1998; 280: 1256–1258.
3. van Arendonk JA, Bijma P. Factors affecting commercial application of embryo technologies in dairy cattle in Europe—a modelling approach. Theriogenology 2003; 59:635–649.
4. Kinghorn BP. Animal production and breeding systems to exploit cloning technology. 2002.
5. Wells DN, Laible G, Tucker FC, Miller AL, Oliver JE, Xiang T, Forsyth JT, Berg MC, Cockrem K, L'Huillier PJ, Tervit HR, Oback B. Coordination between donor cell type and cell cycle stage improves nuclear cloning efficiency in cattle. Theriogenology 2003; 59:45–59.
6. Campbell KH, Ritchie WA, Wilmut I. Nuclear-cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos: implications for deoxyribonucleic acid replication and development. Biol Reprod 1993; 49:933–942.
7. Kurosaka S, Nagao Y, Minami N, Yamada M, Imai H. Dependence of DNA synthesis and in vitro development of bovine nuclear transfer embryos on the stage of the cell cycle of donor cells and recipient cytoplasts. Biol Reprod 2002; 67:643–647.
8. Miyoshi K, Rzucidlo SJ, Pratt SL, Stice SL. Improvements in cloning efficiencies may be possible by increasing uniformity in recipient oocytes and donor cells. Biol Reprod 2003; 68:1079–1086.
9. Bruggerhoff K, Zakhartchenko V, Wenigerkind H, Reichenbach HD, Prelle K, Schernthaner W, Alberio R, Kuchenhoff H, Stojkovic M, Brem G, Hiendleder S, Wolf E. Bovine somatic cell nuclear transfer using recipient oocytes recovered by ovum pick-up: Effect of maternal lineage of oocyte donors. Biol Reprod 2002; 66:367–373.
10. Galli C, Lagutina I, Vassiliev I, Duchi R, Lazzari G. Comparison of microinjection (piezo-electric) and cell fusion for nuclear transfer success with different cell types in cattle. Cloning Stem Cells 2002; 4: 189–196.
11. Hawk HW, Wall RJ. Improved yields of bovine blastocysts from in vitro-produced oocytes. II. Media and co-culture cells. Theriogenology 1994; 41:1585–1594.
12. Keefer CL, Stice SL, Dobrinsky J. Effect of follicle-stimulating hormone and luteinizing hormone during bovine in vitro maturation on

development following in vitro fertilization and nuclear transfer. Mol Reprod Dev 1993; 36:469–474.
13. Barnes F, Endebrock M, Looney C, Powell R, Westhusin M, Bondioli K. Embryo cloning in cattle: the use of in vitro matured oocytes. J Reprod Fertil 1993; 97:317–320.
14. Arat S, Gibbons J, Rzucidlo SJ, Respess DS, Tumlin M, Stice SL. In vitro development of bovine nuclear transfer embryos from transgenic clonal lines of adult and fetal fibroblast cells of the same genotype. Biol Reprod 2002; 66:1768–1774.
15. Roh S, Shim H, Hwang WS, Yoon JT. In vitro development of green fluorescent protein (GFP) transgenic bovine embryos after nuclear transfer using different cell cycles and passages of fetal fibroblasts. Reprod Fertil Dev 2000; 12:1–6.
16. Hill JR, Winger QA, Long CR, Looney CR, Thompson JA, Westhusin ME. Development rates of male bovine nuclear transfer embryos derived from adult and fetal cells. Biol Reprod 2000; 62:1135–1140.
17. Zakhartchenko V, Mueller S, Alberio R, Schernthaner WG, Stojkovic M, Wenigerkind H, Wanke R, Lassnig C, Mueller M, Wolf E, Brem G. Nuclear transfer in cattle with nontransfected and transfected fetal or cloned transgenic fetal and postnatal fibroblasts. Mol Reprod Devel 2001; 60:362–369.
18. Daniels R, Hall VJ, French AJ, Korfiatis NA, Trounson AO. Comparison of gene transcription in cloned bovine embryos produced by different nuclear transfer techniques. Mol Reprod Dev 2001; 60:281–288.
19. Fissore RA, Pinto-Correia C, Robl JM. Inositol trisphosphate-induced calcium release in the generation of calcium oscillations in bovine eggs. Biol Reprod 1995; 53:766–774.
20. Tanaka H, Kanagawa H. Influence of combined activation treatments on the success of bovine nuclear transfer using young or aged oocytes. Anim Reprod Sci 1997; 49:113–123.
21. Choi YH, Lee BC, Lim JM, Kang SK, Hwang WS. Optimization of culture medium for cloned bovine embryos and its influence on pregnancy and delivery outcome. Theriogenology 2002; 58:1187–1197.
22. Iannaccone P, Taborn G, Garton R. Preimplantation and postimplantation development of rat embryos cloned with cumulus cells and fibroblasts. Zygote 2001; 9:135–143.
23. Heindryckx B, Rybouchkin A, Van Der EJ, Dhont M. Effect of culture media on in vitro development of cloned mouse embryos. Cloning 2001; 3:41–50.
24. Oback B, Wiersema AT, Gaynor G, Laible G, Tucker FC, Oliver JE, Miller AL, Troskie HE, Wilson KL, Forsyth JT, Berg MC, Cockrem K, McMillian V, Tervit HR, Wells DN. Cloned cattle derived from a novel zona-free embryo reconstruction system. Cloning Stem Cells 2003; 5:3–12.
25. Peura TT. Improved in vitro development rates of sheep somatic nuclear transfer embryos by using a reverse-order zona-free cloning method. Cloning Stem Cells 2003; 5:13–24.
26. Vajta G, Lewis IM, Trounson AO, Purup S, Maddox-Hyttel P, Schmidt M, Pedersen HG, Greve T, Callesen H. Handmade somatic cell cloning in cattle: analysis of factors contributing to high efficiency in vitro. Biol Reprod 2003; 68:571–578.
27. Kato Y, Tani T, Tsunoda Y. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. J Reprod Fertil 2000; 120:231–237.
28. Jeanisch R, Eggan K, Humpherys D, Rideout W, Hochedlinger K. Nuclear cloning, stem cells, and genomic reprogramming. Cloning Stem Cells 2002; 4:389–396.
29. Arat S, Rzucidlo SJ, Gibbons J, Miyoshi K, Stice SL. Production of transgenic bovine embryos by transfer of transfected granulosa cells into enucleated oocytes. Mol Reprod Dev 2001; 60:20–26.
30. Gao S, Chung YG, Williams JW, Riley J, Moley K, Latham KE. Somatic cell-like features of cloned mouse embryos prepared with cultured myoblast nuclei. Biol Reprod 2003; 69:48–56.
31. Zawada WM, Cibelli JB, Choi PK, Clarkson ED, Golueke PJ, Witta SE, Bell KP, Kane J, Ponce de Leon FA, Jerry DJ, Robl JM, Freed CR, Stice SL. Somatic cell cloned transgenic bovine neurons for transplantation in parkinsonian rats. Nat Med 1998; 4:569–574.
32. Inoue K, Ogonuki N, Mochida K, Yamamoto Y, Takano K, Kohda T, Ishino F, Ogura A. Effects of donor cell type and genotype on the efficiency of mouse somatic cell cloning. Biol Reprod 2003.
33. Gao SR, McGarry M, Ferrier T, Pallante B, Gasparrini B, Fletcher J, Harkness L, de Sousa P, McWhir J, Wilmut I. Effect of cell confluence on production of cloned mice using an inbred embryonic stem cell line. Biol Reprod 2003; 68:595–603.
34. Hill JR, Winger QA, Burghardt RC, Westhusin ME. Bovine nuclear transfer embryo development using cells derived from a cloned fetus. Anim Reprod Sci 2001; 67:17–26.
35. Clark AJ, Ferrier P, Aslam S, Burl S, Denning C, Wylie D, Ross A, de Sousa P, Wilmut I, Cui W. Proliferative lifespan is conserved after nuclear transfer. Nat Cell Biol 2003; 5:535–538.
36. Kasinathan P, Knott JG, Moreira PN, Burnside AS, Jerry DJ, Robl JM. Effect of fibroblast donor cell age and cell cycle on development of bovine nuclear transfer embryos in vitro. Biol Reprod 2001; 64:1487–1493.
37. Kerr DE, Plaut K, Bramley AJ, Williamson CM, Lax AJ, Moore K, Wells KD, Wall RJ. Lysostaphin expression in mammary glands confers protection against staphylococcal infection in transgenic mice. Nat Biotechnol 2001; 19:66–70.
38. Wells KD, Powell A. Blastomeres from somatic cell nuclear transfer embryos are not allocated randomly in chimeric blastocysts. Cloning 2000; 2:9–22.
39. Hawk HW, Wall RJ. Improved yields of bovine blastocysts from in vitro-produced oocytes. I. Selection of oocytes and zygotes. Theriogenology 1994; 41:1571–1583.
40. Xu KP, Yadav BR, Rorie RW, Plante L, Betteridge KJ, King WA. Development and viability of bovine embryos derived from oocytes matured and fertilized in vitro and co-cultured with bovine oviducal epithelial cells. J Reprod Fertil 1992; 94:33–43.
41. Rosenkrans CF Jr, First NL. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. J Anim Sci 1994; 72:434–437.
42. Wakayama T, Yanagimachi R. Mouse cloning with nucleus donor cells of different age and type. Mol Reprod Devel 2001; 58:376–383.
43. Balin AK, Fisher AJ, Anzelone M, Leong I, Allen RG. Effects of establishing cell cultures and cell culture conditions on the proliferative life span of human fibroblasts isolated from different tissues and donors of different ages. Exp Cell Res 2002; 274:275–287.
44. Brophy B, Smolenski G, Wheeler T, Wells D, L'Huillier P, Laible G. Cloned transgenic cattle produce milk with higher levels of beta-casein and kappa-casein. Nat Biotechnol 2003; 21:157–162.
45. Tani T, Kato Y, Tsunoda Y. Developmental potential of cumulus cell-derived cultured cells frozen in a quiescent state after nucleus transfer. Theriogenology 2000; 53:1623–1629.
46. Cui W, Wylie D, Aslam S, Dinnyes A, King T, Wilmut I, Clark AJ. Telomerase-immortalized sheep fibroblasts can be reprogrammed by nuclear transfer to undergo early development. Biol Reprod 2003; 69:15–21.
47. Booth PJ, Viuff D, Tan S, Holm P, Greve T, Callesen H. Numerical chromosome errors in day 7 somatic nuclear transfer bovine blastocysts. Biol Reprod 2003; 68:922–928.
48. Booth PJ, Tan SJ, Reipurth R, Holm P, Callesen H. Simplification of bovine somatic cell nuclear transfer by application of a zona-free manipulation technique. Cloning Stem Cells 2001; 3:139–150.
49. Peura TT, Trounson AO. Recycling bovine embryos for nuclear transfer. Reprod Fertil Dev 1998; 10:627–632.
50. Campbell KH, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. Nature 1996; 380:64–66.
51. Kuhholzer B, Hawley RJ, Lai L, Kolber-Simonds D, Prather RS. Clonal lines of transgenic fibroblast cells derived from the same fetus result in different development when used for nuclear transfer in pigs. Biol Reprod 2001; 64:1695–1698.
52. Parker L, Fuehr K. Low oxygen concentration extends the lifespan of cultured human diploid cells. Nature 1977; 267:423–425.
53. von Zglinicki T, Saretzki G, Docke W, Lotze C. Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence? Exp Cell Res 1995; 220:186–193.
54. Richter A, Sanford KK, Evans VJ. Influence of oxygen and culture media on plating efficiency of some mammalian tissue cells. J Natl Cancer Inst 1972; 49:1705–1712.
55. Taylor WG, Richter A, Evans VJ, Sanford KK. Influence of oxygen and pH on plating efficiency and colony development of WI-38 and Vero cells. Exp Cell Res 1974; 86:152–156.
56. Richter A, Sanford KK, Evans VJ. Influence of oxygen and culture media on plating efficiency of some mammalian tissue cells. J Natl Cancer Inst 1972; 49:1705–1712.
57. Talbot N, Powell A, Caperna T. Comparison of colony-formation efficiency of bovine fetal fibroblast cell lines cultured with low oxygen, hydrocortisone, l-carnosine, bFGF or different levels of FBS. Clon Stem Cells 2003; (in press).